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BASIC SCIENCE
Lipids

A common variant in *CCDC93* protects against myocardial infarction and cardiovascular mortality by regulating endosomal trafficking of low-density lipoprotein receptor

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Aims Genome-wide association studies have previously identified *INSIG2* as a candidate gene for plasma low-density lipoprotein cholesterol (LDL-c). However, we suspect a role for *CCDC93* in the same locus because of its involvement in the recycling of the LDL-receptor (LDLR).

Methods and results Characterization of the *INSIG2* locus was followed by studies in over 107 000 individuals from the general population, the Copenhagen General Population Study and the Copenhagen City Heart Study, for associations of genetic variants with plasma lipids levels, with risk of myocardial infarction (MI) and with cardiovascular mortality. *CCDC93* was furthermore studied in cells and mice. The lead variant of the *INSIG2* locus (rs10490626) is not associated with changes in the expression of nearby genes but is a part of a genetic block, which excludes *INSIG2*. This block includes a coding variant in *CCDC93* p.Pro228Leu, which is in strong linkage disequilibrium with rs10490626 (r^2 > 0.96). In the general population, separately and combined, *CCDC93* p.Pro228Leu is dose-dependently associated with lower LDL-c (*P*-trend 2.5 × 10^{-6} to 8.0 × 10^{-9}), with lower risk of MI (*P*-trend 0.04–0.002) and lower risk of cardiovascular mortality (*P*-trend 0.005–0.004). These results were validated for LDL-c, risk of both coronary artery disease and MI in meta-analyses including from 194 000 to >700 000 participants. The variant is shown to increase CCDC93 protein stability, while overexpression of human CCDC93 decreases plasma LDL-c in mice. Conversely, CCDC93 ablation reduces LDL uptake as a result of reduced LDLR levels at the cell membrane.

Conclusion This study provides evidence that a common variant in *CCDC93*, encoding a protein involved in recycling of the LDLR, is associated with lower LDL-c levels, lower risk of MI and cardiovascular mortality.

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Keywords Low-density lipoprotein cholesterol • Myocardial infarction • Cardiovascular mortality • LDL-receptor • Endosomal trafficking • *CCDC93* • CCC complex

Translational perspective

The present study shows that a common genetic variant in the gene *CCDC93* is associated with lower plasma low-density lipoprotein cholesterol (LDL-c) levels and a corresponding lower risk of myocardial infarction in the general population. This study suggests that endosomal trafficking and/or recycling of LDL-receptor could be a target for pharmaceutical intervention.

Introduction

Atherosclerotic cardiovascular disease is a major cause of morbidity and mortality[1] for which low-density lipoprotein cholesterol (LDL-c) is a well-established causal risk factor.[2–5] Genome-wide association studies have identified numerous regions in the genome (loci) that are primarily associated with plasma LDL-c concentration.[6–10] For approximately half of the LDL-c associated loci, it is not known which mechanisms underlie these associations, emphasizing that our knowledge of LDL metabolism is far from complete.

Here, we focus on the single nucleotide polymorphism (SNP) rs10490626, on the second chromosome at position 118835841 (GRCh37). The T allele is found in ~9% of non-Finnish Europeans (http://gnomad.broadinstitute.org) and has been reported to be associated with reduced plasma LDL-c levels.[9,10] This variant is located in the locus 2q14 upstream of the INSulin Induced Gene 2 (*INSIG2*) which is implicated in the regulation of cholesterol synthesis. Loss of *Insig2* does, however, not affect plasma cholesterol levels in mice.[11] This, combined with recent insight, led us to suspect a role for coiled-coil domain-containing protein 93 (*CCDC93*), another gene in this locus. This because we have recently shown that CCDC93 participates in an evolutionary conserved multiprotein complex, annotated as the CCC complex (COMMD/CCDC22/CCDC93; see Supplementary material online for additional information), that orchestrates the endosomal recycling of the LDL-receptor (LDLR).[12,13] Loss of proteins of the CCC complex studied thus far causes hypercholesterolaemia in mammals including man.

The current study contributes to the unravelling of a mechanism that offers an explanation of how rs10490626 is associated with reduced plasma levels of LDL-c and protection against myocardial infarction (MI) and death from cardiovascular disease. Several lines of evidence suggest that stabilization of the endosomal sorting machinery through a coding variant of CCDC93 increases LDLR recycling, thereby lowering levels of LDL-c in plasma.

Methods

A flowchart of epidemiological and genetic analyses is shown in Supplementary material online, *Figure S1*. A brief description of the methods is provided below. For more detailed descriptions refer Supplementary material online, *Appendix*.

Characterization of the chromosomal region 2q14

Expression quantitative trait loci (eQTL) analyses aim to connect SNPs with the expression of genes. We first tested whether the T allele of rs10490626 was associated with expression levels of protein-coding genes in the locus 2q14 (within 1 Mb surrounding this SNP) using a publicly available mRNA dataset (GTEx; V7 release).[14] In addition, we used an eQTL dataset from Biobank-based Integrative Omics Studies (BIOS) Consortium of the BBMRI-NL (http://wiki.bbmri.nl/wiki/BIOS_bios) which is composed of whole blood samples from 2116 donors.

Pairwise linkage disequilibrium (LD) values were obtained from the Phase 3 release of the 1000 Genomes project.[15] In addition, LD between rs10490626 and rs17512204 (*CCDC93* p.Pro228Leu) was determined in individuals in the studied populations (see below).

Participants

We included individuals from two prospective studies of the Danish general population, The Copenhagen General Population Study (CGPS) and The Copenhagen City Heart Study (CCHS) that have been described in detail elsewhere.[16] All individuals were white and of Danish descent.

Combining the participants in the CGPS and CCHS yielded a total of 107 063 participants at baseline. During a mean follow-up of 36 years (range: <1–40 years) which ended in March 2017, MI developed in 5291 individuals and 3635 died from ischaemic cardiovascular disease. In both studies, follow-up was 100% complete, i.e. we did not lose track of a single individual. DNA was available for all individuals, and lipid values were available for more than 98%.

The study was approved by institutional review boards and Danish ethics committees and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all individuals. For additional information on participation rates, clinical endpoints, laboratory analyses, and other covariates (Supplementary material online, *Appendix*).

Genotyping and statistical analyses

CCDC93 p.Pro228Leu (rs17512204; C>T) which is in complete LD with rs10490626 (see Results section), was genotyped using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) and TaqMan-based assays.

Data were analysed using Stata/S.E. 14.0. The χ^2 test evaluated Hardy–Weinberg equilibrium. To compare baseline characteristics by *CCDC93* p.Pro228Leu (rs17512204) genotype, we used Cuzick's test for trend. For trend tests, genotypes were coded 0, 1, and 2. The genotype associated with the highest LDL-c levels was used as the reference (coded 0). *P*-values were by tests for trend from linear regression models. Cox proportional hazards regression models, using age as time scale and delayed entry (left truncation) which implies that age is automatically adjusted for, were used to estimate hazard ratios for MI and cardiovascular mortality as a function of *CCDC93* p.Pro228Leu (rs17512204) genotypes. Multifactorial adjustments were for well-known risk factors for MI: age, gender, body mass index, hypertension, diabetes mellitus, physical activity, smoking, alcohol consumption, hormone replacement therapy

(women only), and lipid-lowering therapy. Analyses were conducted from 1 January 1977 (start of the national Danish Patient Registry and the national Danish Causes of Death Registry) through March 2017.

Finally, we performed meta-analyses of *CCDC93* rs17512204 (p.Pro228Leu) on LDL-c, risk of coronary artery disease (CAD) and MI combining data from the Danish general population with data from: (i) the Global Lipids Genetics Consortium (Ref.[9] and http://lipidgenetics.org) for LDL-c; (ii) summary data from the UK Biobank (http://geneatlas.roslin.ed.ac.uk) and CARDIoGRAMplusC4D 1000 Genomes (Ref.[17] and http://www.cardiogramplusc4d.org/data-downloads/) for risk of ischaemic heart disease and MI using the 'metan' command in Stata.

In vitro characterization of *CCDC93* Pro228Leu

FLAG-tagged *CCDC93* (pcDNA3.1-CCDC93-FLAG) was purchased from Genscript (OHu01865D). pcDNA3.1-*CCDC93*-p.Pro228Leu-FLAG was generated with the QuikChange II Site-Directed Mutagenesis Kit. Wildtype (CCCD93WT) and CCDC93 p.Pro228Leu were transiently overexpressed in HEK293T cells. The protein stability of both variants was assessed after blocking protein synthesis at an optimal concentration of cycloheximide that did not affect cell viability, i.e. 50 µg/mL (CHX – Sigma C4859). In short, 24 h after transfection, cells were cultured in medium supplemented with cycloheximide and harvested at sequential time points (from 4 to 36 h). Cell lysates were used for western blotting using anti-FLAG antibody. Statistical comparison of protein concentrations between CCDC93WT vs CCDC93p.Pro228Leu was calculated using the Student's *t*-test.

Overexpression of human CCDC93 in Ldlr$^{+/-}$ mice

Liver overexpression of human CCDC93 (hCCDC93) in *Ldlr*$^{+/-}$ mice ($n = 5$) was achieved through intravenous administration of adeno-associated virus 8 (AAV8) harbouring hCCDC93, or with a matched viral dose of empty AAV8 particles as controls ($n = 5$). Animals were single housed and fed a standard chow diet. Three weeks after injections, animals were fasted and sacrificed by cardiac puncture under anaesthesia. Plasma cholesterol concentrations in the main lipoprotein classes [very low-density lipoprotein, LDL, and high-density lipoprotein (HDL)] were determined using fast protein liquid chromatography in plasma of each animal and compared using the Student's *t*-test. This study was approved by the Institutional Animal Care and Use Committee, University of Groningen (the Netherlands).

Generation and characterization of a CCDC93 deficient liver cell line

Huh7 CCDC93 knockout cells (*CCDC93*$^{-/-}$) were generated using CRISPR/Cas9 editing technology.[18] To quantify protein concentration, we used custom targeted liquid chromatography-mass spectrometry (LC/MS) based proteomic assays.[12,13]

Low-density lipoprotein uptake capacity of the cells [Huh7 *CCDC93*$^{-/-}$ vs. wild-type (wt)] was evaluated using DiI-labelled LDL. Cells were deprived of serum for 16 h and subsequently incubated with DiI-LDL (5 mg/mL) containing medium for 2.5 h. After washing, fixation, and mounting, DiI-LDL uptake was quantified and normalized by fluorescent microscopy in a blinded fashion. More than 700 cells per condition were recorded.

To assess the level of the LDLR at the cell surface, we used a biotinylation assay which targets proteins at the plasma membrane. Briefly, cells were incubated with biotin and lysed. The biotinylated membrane fraction was isolated using neutravidin beads. Both, membrane and whole cell lysate fractions were analysed using western blotting. For both LDL uptake and biotinylation assays, the Student's *t*-test was used to compare different conditions.

Results

Genetic association and characterization of the 2q14 locus

Among SNPs spanning the region of chromosome 2 (locus 2q14) (*Figure 1A*), rs10490626 shows the strongest association (*y* axis, -log10 *P*-value) with LDL-c plasma levels. The respective SNP is located 246 kb downstream of *DDX18*, 64 kb upstream of *CCDC93*, and 10 kb upstream of *INSIG2* (*x* axis, *Figure 1A*). We found that rs10490626 is part of an LD block of 200 kb ($r^2 > 0.8$, data from the Phase 3 release of the 1000 Genomes project[15]) that includes *CCDC93*, but not *INSIG2* and *DDX18* (*Figure 1A*). This LD block includes rs17512204, a SNP that leads to the substitution of a leucine for a proline residue at position 228 of CCDC93 (NM_019044.4: rs17512204_c.683C>T, p.Pro228Leu). Importantly, rs17512204 is in complete LD with rs10490626 ($r^2 = 0.96$, *P*-value: <0.0001; data from 1000 Genomes project) (*Figure 1A*) implying that these SNPs are co-inherited.

Using the GTEx Consortium mRNA dataset (Release V7), we assessed whether rs10490626 is associated with the expression of *DDX18*, *CCDC93*, or *INSIG2*. In line with previous findings,[9] the data show that rs10490626 is probably not an eQTL (plotted association data in adipose, liver, and intestine, Supplementary material online, *Figure S2*). Taken the small number of samples of these relevant tissues ($n = 385$, 313, 153, and 125 for subcutaneous adipose tissue, visceral adipose tissue, liver, and intestine, respectively) combined with the frequency of rs10490626, these eQTL analyses were, however, underpowered. Of note, GTEx suggests that rs10490626 is an eQTL in testis tissue for the pseudogene *HTR5BP*, which is exclusively expressed in testis (data not shown) and is very unlikely relevant in regard to the plasma-cholesterol metabolism.

In a next step, we took advantage of the fact that *DDX18*, *CCDC93*, and *INSIG2* are all ubiquitously expressed [according to GTEx[14] (https://www.gtexportal.org) and The Human Protein Atlas (https://www.proteinatlas.org)[19]] and ran an eQTL analysis using RNA-seq data set of whole human blood ($n = 2116$).[20] *Figure 1B* shows that rs10490626 is not associated with changes in the expression of *DDX18*, ($P = 0.31$), *CCDC93* ($P = 0.17$), or *INSIG2* ($P = 0.94$) in blood, which suggests that rs10490626 is not an eQTL for the genes studied.

CCDC93 genotype, plasma lipids, lipoproteins, and apolipoproteins in the Danish general population cohorts

In agreement with publicly available data, LD between the minor alleles of the intergenic lead GWAS SNP, rs10490626 and rs17512204 (*CCDC93* p.Pro228Leu) was near complete ($r^2 = 0.98$) in both the CGPS and the CCHS population cohorts, thereby validating the use of the latter variant in our study. Baseline characteristics of the 107 063 individuals from the CGPS and CCHS, combined and individually, as a function of *CCDC93* p.Pro228Leu genotype (rs17512204; C>T) are shown in Supplementary material online,

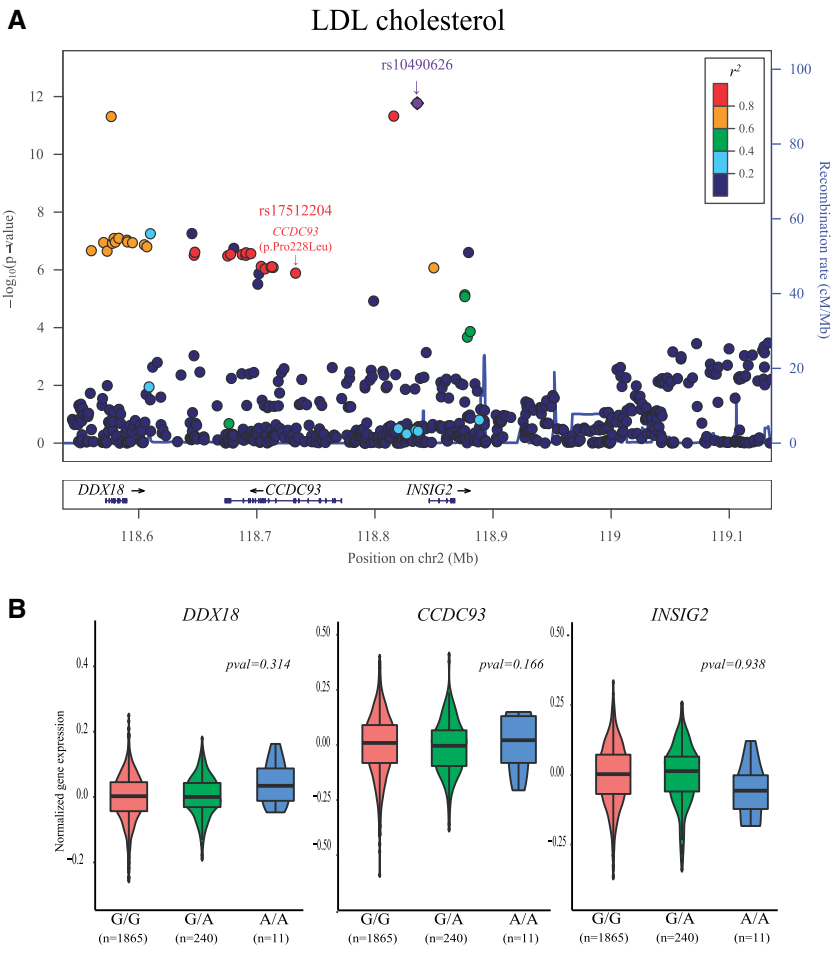


Figure 1 Genetic association with low-density lipoprotein cholesterol and fine mapping of the locus 2q14. (*A*) Illustrates the association with low-density lipoprotein cholesterol (*y* axis) for single nucleotide polymorphisms spanning the region of chromosome 2 including *CCDC93*, *DDX18*, and *INSIG2*, and the pairwise linkage disequilibrium between rs10490626 and other single nucleotide polymorphisms. Red colour indicates strong linkage disequilibrium ($r^2 > 0.8$). As shown, rs10490626 is in complete linkage disequilibrium ($R^2 > 0.80$) with rs17512204 (*CCDC93* p.Pro228Leu) and with other non-coding single nucleotide polymorphisms spanning *CCDC93*.[9] Plots were generated from publicly available data and the online locuszoom tool (http://locuszoom.org/). (*B*) Normalized gene expression for *DDX18*, *CCDC93*, and *INSIG2* as a function of rs10490626 G>A genotypes showing that genotypes are not associated with levels of gene expression within the 2q14 locus: *DDX18* (*P*=0.314), *CCDC93* (*P*=0.166), and *INSIG2* (*P*=0.94) when using eQTL dataset from BIOS-consortium containing 2116 blood samples (*B*).

Tables S1–S3. Characteristics as a function of genotype were similar. In agreement with the corresponding lower LDL-c levels (*Figure 2A*, second panel), the use of lipid-lowering therapy tended to be slightly less frequent as a function of increasing number of T-alleles (*P* for trend < 0.05) (Supplementary material online, *Table S1*). Taken together, these data suggest that the genetic data are largely unconfounded by the main known measured risk factors for MI. Finally, genotype distribution did not differ from Hardy–Weinberg equilibrium.

CCDC93 p.Pro228Leu (rs17512204; C>T) was associated with lower plasma levels of total cholesterol up to 0.9% (-0.05 mmol/L, *P* for trend = 4.8×10^{-8}), LDL-c up to 2.2% (-0.07 mmol/L, *P* for trend = 8.0×10^{-9}), and apolipoprotein B up to 1.0% (-1.14 mg/dL, *P* for trend = 3.4×10^{-6}), in homozygotes for the minor T-allele vs. non-carriers (*Figure 2A*, Panels 1–3 from left) but was not associated with HDL cholesterol, apolipoprotein AI (ApoAI), triglycerides, calculated remnant cholesterol (which mirrors triglycerides), or lipoprotein(a) (*Figure 2A*, Panels 4–7; data not shown for calculated remnant cholesterol). Results were similar for the CGPS and the CCHS separately (Supplementary material online, *Figure S3*). In individuals who were not on lipid-lowering therapy (*N* = 95 512), total cholesterol, LDL-c, and apolipoprotein B were even further reduced (compare *Figure 2A* and Supplementary material online, *Figures S3* with *S4*).

CCDC93 genotype and risk of myocardial infarction

The multifactorially adjusted hazard ratios for MI decreased stepwise as a function of rs17512204 genotype to 0.88 (95% confidence interval: 0.81–0.96) in CT heterozygotes and 0.67(0.39–1.16) in TT homozygotes (*P* for trend = 0.002) (*Figure 2B*). In the individual studies, the corresponding hazard ratios were 0.92 (0.83–1.01) and 0.67 (0.36–

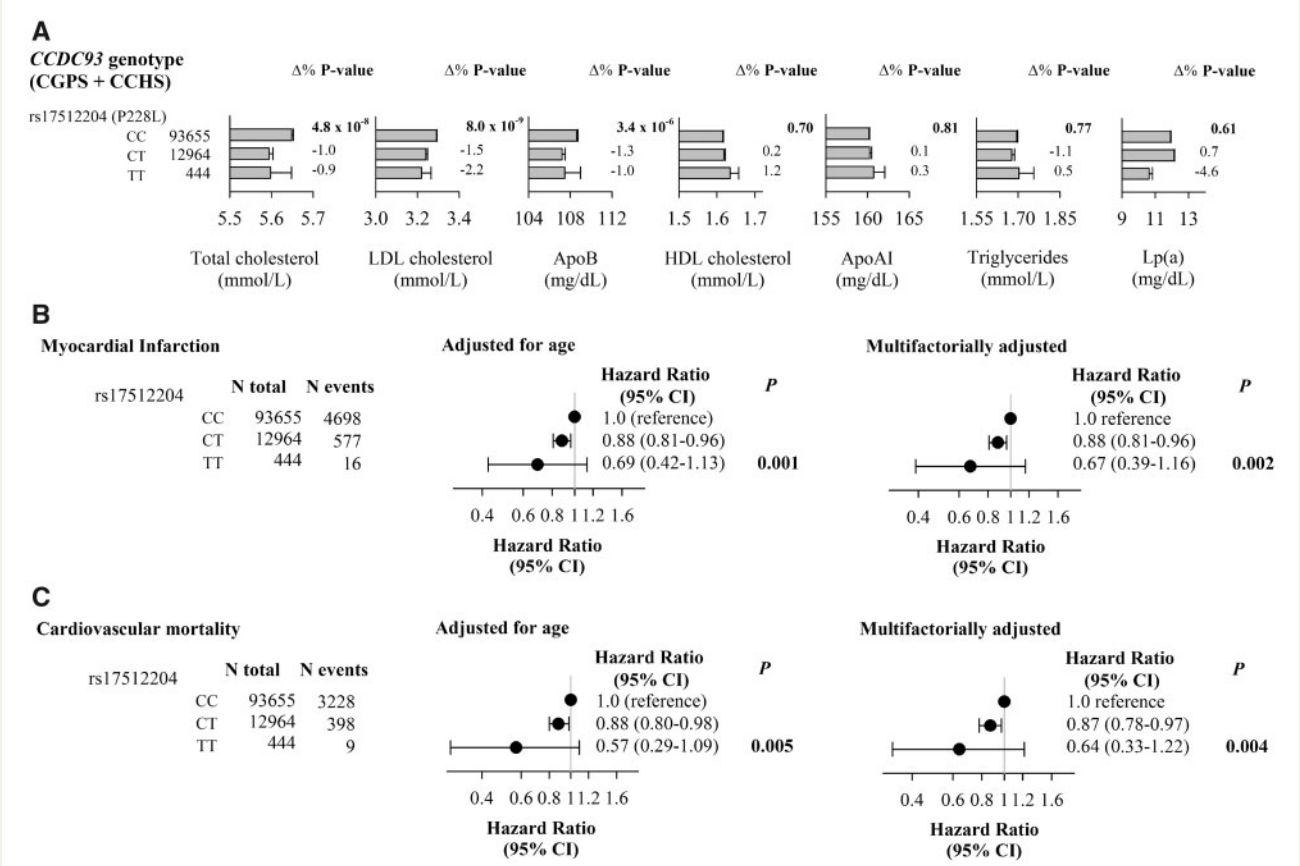


Figure 2 Plasma lipid levels and risk of myocardial infarction and cardiovascular mortality as a function of *CCDC93* P228L (rs17512204; C>T) genotype in the general population. Data are from the Copenhagen General Population Study and the Copenhagen City Heart Study cohorts (combined 107 063 participants). (*A*) *P*-values were tested for trend using linear regression models. (*B* and *C*) Cox proportional hazards regression models using age as time and delayed entry (left truncation) were used to estimate hazard ratios for myocardial infarction and cardiovascular mortality as a function of genotype. Hazard ratios were adjusted for age (left panel) or multifactorially adjusted for age, gender, body mass index, hypertension, diabetes mellitus, physical activity, smoking, alcohol consumption, hormone replacement therapy (women only), and lipid-lowering therapy. *P*-values are for tests for trend for hazard ratios. *N*, number of individuals.

1.24) in the CGPS (*P* for trend = 0.04), and 0.77 (0.63–0.93) and 0.71 (0.23–2.20) in the CCHS (*P* for trend = 0.007) (Supplementary material online, *Figure S5A* and *C*).

CCDC93 genotype and cardiovascular mortality

The multifactorially adjusted risk of cardiovascular mortality (*N* = 4126 events) decreased stepwise as a function of rs17512204 genotype to 0.87 (95% confidence interval: 0.78–0.97) in CT heterozygotes and 0.64 (0.33–1.22) in TT homozygotes (*P* for trend = 0.004) (*Figure 2C*). Results were similar in the CGPS and CCHS separately (*Supplementary Figure S5B* and *D*).

Meta-analyses on low-density lipoprotein cholesterol, risk of ischaemic heart disease, and myocardial infarction

In meta-analyses of *CCDC93* rs17512204 (p.Pro228Leu) on LDL-c including data from the Global Lipids Genetics Consortium (Ref.[9] and http://lipidgenetics.org) and the Danish general population (CGPS + CCHS), the per T-allele reduction in LDL-c was -0.05 standard deviation for the inversed normalized distribution of LDL-c (95% CI: -0.06 to -0.04) using both random and fixed effects models (*Figure 3A*), corresponding to an approximate reduction of 0.05 mmol/L (2 mg/dL) in our study. There was no evidence of heterogeneity between studies (I^2 = 0%, *P* = 0.84).

In meta-analysis of risk of CAD, the per T-allele odds ratio was 0.97 (0.94–0.99) in both random and fixed effects models (*Figure 3B*). CAD was self-reported heart attack or MI in the UK Biobank. In the Danish general population, this diagnosis corresponded best to ischaemic heart disease and included angina pectoris and MI based on International Classification of Diseases codes (ICD8: 410–414 and ICD10: I20–25); MI was independently validated—for a detailed description see Supplementary material online, *Methods*. In CARDIoGRAMplusC4D 1000 Genomes (Ref.[17] and http://www.cardiogramplusc4d.org/data-downloads/), CAD was based on diagnostic criteria in 48 different studies which were harmonized across studies (for a detailed description of diagnoses in the individual studies see

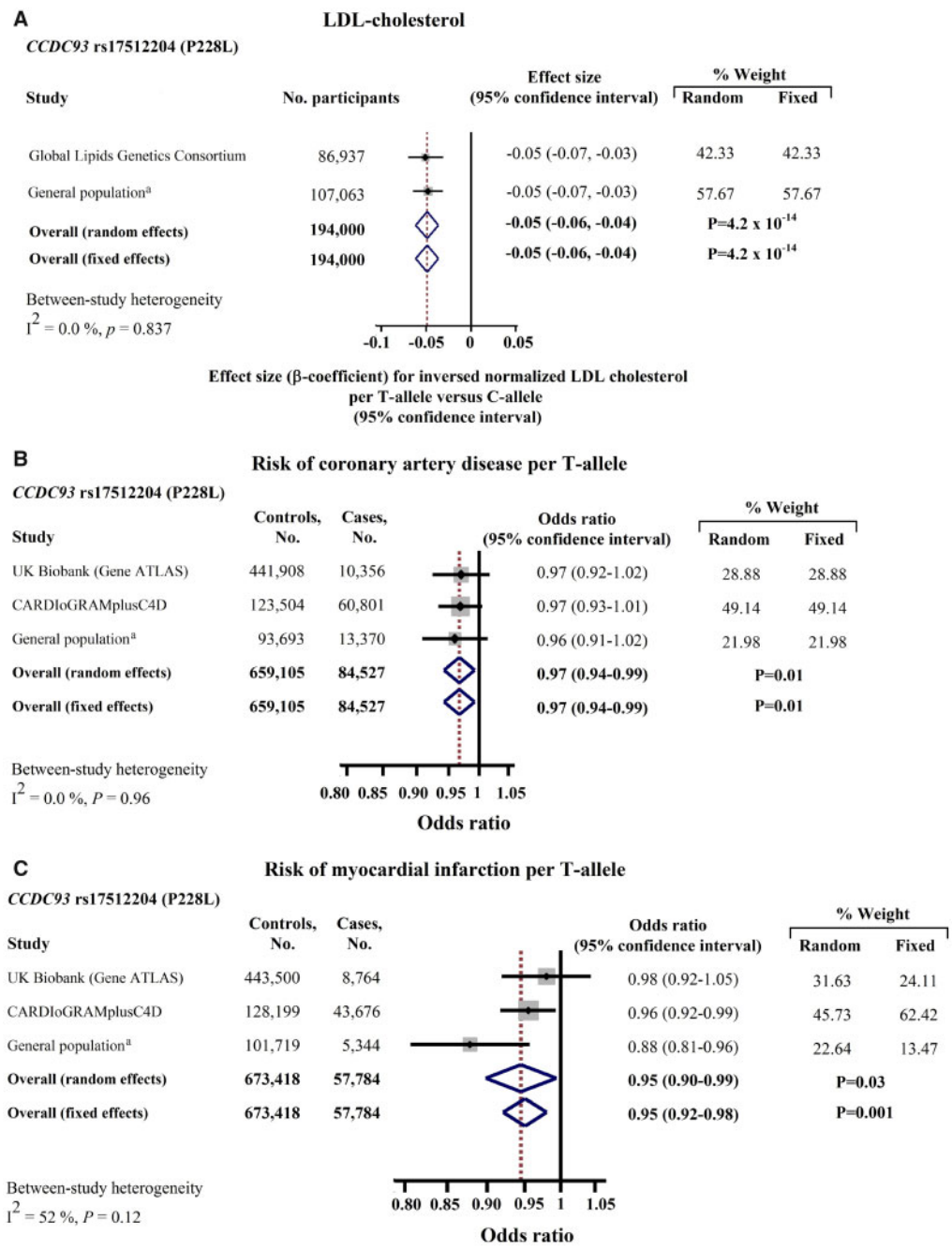


Figure 3 Meta-analyses of low-density lipoprotein cholesterol, risk of coronary artery disease, and myocardial infarction per rs17512204 C>T (*CCDC93* p.P228L) T-allele. (*A*) Meta-analysis summarizing effect size on low-density lipoprotein cholesterol per T-allele. Global Lipids Genetics consortium (Ref.[9] and http://lipidgenetics.org/). (*B*) Meta-analysis summarizing risk of coronary heart disease per T-allele. CARDIoGRAMplusC4D 1000 Genomes (Ref.[17] and http://www.cardiogramplusc4d.org/data-downloads/); UK Biobank summary data from GeneAtlas (http://geneatlas.roslin.ed.ac.uk). The diagnosis coronary artery disease was self-reported heart attack/myocardial infarction in the UK Biobank (http://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=20002), coronary artery disease from CARDIoGRAMplusC4D 1000 Genomes (data as published in Ref.[17]) and ischaemic heart disease (including angina pectoris based on ICD code, and myocardial infarction based on ICD code and independently validated to a validity >99%). (*C*) Meta-analysis summarizing risk of myocardial infarction per T-allele. Myocardial infarction was myocardial infarction in the UK Biobank (ICD10 code I21 acute myocardial infarction, http://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=41202; summary data from http://geneatlas.roslin.ed.ac.uk) myocardial infarction from sub-phenotype analysis in CARDIoGRAMplusC4D, and myocardial infarction (ICD8: 410 and ICD10 I21 and I22; validated to a validity of >99% by cardiologists). In all three meta-analyses, horizontal lines correspond to 95% confidence intervals by forest plots. Diamonds and red broken vertical lines represent the summary estimates. The width of the diamonds represents the confidence intervals for the summary estimates. Grey shaded areas correspond to the weight of the study; weights on the graphs are from random effects models. Overall effect sizes are Cohen's d with 95% confidence intervals. [a]Copenhagen General Population Study and Copenhagen City Heart Study combined.

Ref.[17] Supplementary material online, *Notes*). There was no evidence of heterogeneity between studies (I^2=0%, P = 0.96) (*Figure 3B*).

In meta-analysis of MI, the overall odds ratio for MI per T-allele was 0.95 (0.90–0.99) using a random-effects model, and 0.95 (0.92–0.98) using a fixed-effects model (*Figure 3C*). There was modest, non-significant heterogeneity between studies (I^2 = 53%, P = 0.12) with overlapping confidence intervals. The estimates became significant for both CARDIoGRAMplusC4D 1000 Genomes [0.96 (0.92–0.99)] and our study [0.88 (0.81–0.96)], but not different from unity for the UK Biobank, on the hard endpoint MI.

Sensitivity analyses

To determine whether *CCDC93* p.Pro228Leu affected other phenotypes than LDL-c which might additionally explain the effect on risk of CAD, MI, and cardiovascular mortality, or affect other endpoints, we first determined the per T-allele effect on 34 different phenotypes and an additional five endpoints in our own study (Supplementary material online, *Figure S6*). Only total cholesterol, LDL-c, apolipoprotein B, and risk of MI were significant. Second, we collected all available data from GWAS studies and chip data from the Cardiovascular Disease Knowledge Portal (29 March 2019; http://broadcvdi.org/variantInfo/variantInfo/rs17512204) (for a description of the studies included, see legend to Supplementary material online, *Figure S7*). In these meta-analyses including 30 phenotypes or endpoints, the per T-allele effect reached genome-wide significance for total cholesterol and LDL-c only (Supplementary material online, *Figure S7*). Third, we included summary 'phewas' (phenomewide association study) data from the UK Biobank for rs17512204 (http://geneatlas.roslin.ed.ac.uk). In that study, 'high cholesterol' was the most significant hit (note that the reference allele in the UK Biobank was the wild-type G-allele, implying that the results are opposite using the minor T-allele as the reference in our study, i.e. low cholesterol), while there were indications that the variant might affect anthropometric parameters (for T-allele: increased leg fat mass, increased weight and hip circumference, but also increased basic metabolic rate, and lower physical activity) (Supplementary material online, *Table S4*). Combined, these latter findings are unlikely to reduce risk of CAD.

Proportional risk of coronary artery disease and myocardial infarction as a function of per allele genetically determined increase in low-density lipoprotein cholesterol in the general population (CCHS and CGPS)

The proportional risk of CAD and MI as a function of the per allele genetically determined increase in LDL-c for variants in well-known LDL-c genes (*NPC1L1, PCSK9, ABCG8, APOB*, and the *LDLR*) in the CCHS and CGPS is shown in Supplementary material online, *Figure S8* and compared to the effect for *CCDC93* p.Pro228Leu (G-allele). First, the proportional risk of CAD and MI for *CCDC93* p.Pro228Leu (G-allele) was comparable to that of other LDL-c genes. Second, per allele risk of CAD for genetic variants in these genes was similar to risks reported in the EAS consensus paper. These data together with data from the meta-analyses suggest that *CCDC93* p.Pro228Leu is likely associated with risk of CAD and MI through the LDL-c effect *per se*. Finally as expected, risk of MI, a more valid endpoint, was higher than risk of CAD for most variants in our study, and therefore, these two endpoints are not directly comparable, as also shown for CARDIoGRAMplusC4D 1000 Genomes in the meta-analyses (*Figure 3*).

CCDC93 p.Pro228Leu increases protein stability

In a next step, we investigated whether the substitution of a Leucine for a Proline residue at position 228 in the CCDC93 protein could explain our genetic and epidemiologic observations. To this purpose, we transiently overexpressed wildtype (WT) and the p.Pro228Leu variant in HEK293T cells and investigated their half-life ($t_{1/2}$) following incubation with a protein synthesis inhibitor (cycloheximide) (*Figure 4A* and *B*). We found that the protein stability of CCDC93 p.Pro228Leu was significantly increased compared to CCDC93WT after 24 and 36 h (P < 0.05) (*Figure 4A* and *B*). This finding suggests that CCDC93 p.Pro228Leu increases the stability of CCDC93. Analysis of the whole CCC complex with LC-MS based targeted proteomics furthermore shows that CCDC93 p.Pro228Leu does not affect the stoichiometry of CCC complex (Supplementary material online, *Figure S9*).

Increased expression of human CCDC93 in the liver reduces low-density lipoprotein cholesterol plasma levels in mice

The increased protein stability of CCDC93 p.Pro228Leu and its association with reduced plasma LDL-c led us to evaluate whether increasing the levels of wild-type CCDC93 in the liver of mice could reduce plasma LDL-c. To this end, we injected heterozygous *Ldlr* knockout mice (C57Bl6; Jackson Labs) with AAV8 harbouring wild-type human (h) *CCDC93*. Three weeks after virus administration, we observed a 1.8-fold increase of CCDC93 protein in the liver compared to controls (*Figure 5A*). This was associated with a small but significant reduction of LDL-c (P = 0.049) in mice overexpressing h*CCDC93* compared to controls (*Figure 5B*; Supplementary material online, *Table S5*). Using LC-MS based targeted proteomics, we confirmed that CCD93 protein concentration was increased (1.6 fold) (P < 0.001) in liver homogenates, which was accompanied by an increase of the CCC complex component CCDC22 (P < 0.001) in mice that were treated with AAV8 h*CCDC93* compared to controls (Supplementary material online, *Figure S10*). Although the overexpression of the WT form of *CCDC93* does not mimic a gain of function mutation, these results support the idea that increasing the amount of hepatic CCDC93 can lower plasma LDL-c.

CCDC93 is required for low-density lipoprotein receptor recycling and low-density lipoprotein uptake in liver cells

To validate the role of CCDC93 on endosomal LDLR recycling, we ablated CCDC93 in human hepatocyte carcinoma cells (Huh7) (*Figure 6A*). While this cell line cannot recapitulate all aspects of human lipid metabolism, the involvement of the CCC complex in facilitating endosomal trafficking of LDLR has also been shown in HEK293T, HepG2 as well as primary murine hepatocytes and several animal models.[12,13] Loss of CCDC93 decreased the protein levels of

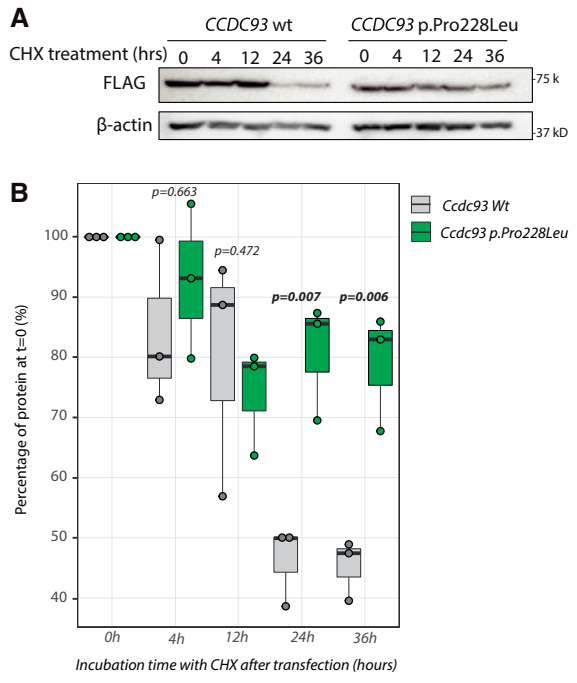


Figure 4 *In vitro* characterization of the variant *CCDC93* Pro228leu. HEK293T cells transiently overexpressing CCDC93WT or CCDC93p.Pro228Leu were incubated for 0, 4, 12, 24, and 36 h with an inhibitor of protein synthesis (cycloheximide). Cell lysates were analysed by western blotting (*A*) ($n = 3$). Protein degradation was quantified, normalized against Beta actin as loading control and plotted using $t = 0$ as reference ($t = 0$: 100%) for each condition *B*. The data show that both CCDC93 variants are relatively stable until 12 h, however, CCDC93WT levels drop after 24h (∼40% decrease), whereas CCDC93p.Pro228Leu stays relatively stable beyond 36 h after transfection (∼85% of the initial protein amount). Statistical comparison was carried out using the Student's *t*-test.

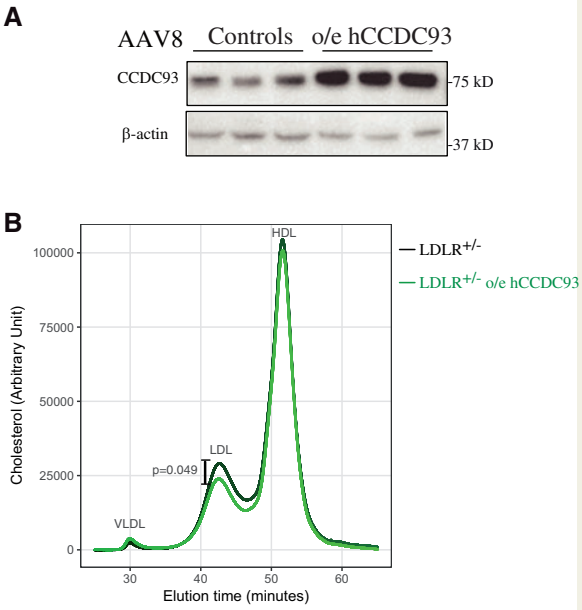


Figure 5 Overexpression of CCDC93 in mice reduces low-density lipoprotein cholesterol plasma levels. Overexpression of human (h)CCDC93 in *Ldlr*$^{+/-}$ mice was achieved through intravenous administration of adeno-associated virus 8. Mice were either injected with AAV8 harbouring human (h)CCDC93 (AAV8-hCCDC93) ($n = 5$) or AAV8 empty as controls ($n = 5$). (*A*) Protein overexpression of CCDC93 (1.8-fold increase on average) in the liver of mice injected with AAV8-hCCDC93 (o/e hCCDC93) compared to controls. Main plasma lipoprotein classes were separated using fast protein liquid chromatography (*x* axis fast protein liquid chromatography fractions) for each individual mouse. Plasma cholesterol concentration (*y* axis in arbitrary units) was measured in each of the lipoprotein classes. The fractions were determined as follow: VLDL = fractions from 28 to 34 min; LDL = fractions from 34 to 47 min; HDL = fractions from 47 to 60 min. Mice overexpressing hCCDC93 in the liver present a decrease of low-density lipoprotein cholesterol compared to controls ($P = 0.049$; *B*). HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL-receptor; VLDL, very low-density lipoproteins.

the CCC core components CCDC22 and C16orf62 which is in line with previous observations[12,13] (*Figure 6B*). This destabilization of the CCC complex was accompanied by a ∼50% reduction of LDLR at the plasma membrane ($P < 0.01$) (*Figure 6C*) and a ∼40% reduction in the uptake of fluorescently labelled LDL (DiI-LDL) compared to control cells ($P < 0.001$) (*Figure 6D*). Combined, these results suggest that CCDC93 is involved in the endosomal trafficking of LDLR back to the plasma membrane for the uptake of LDL (*Figure 6E*).

Discussion

This study suggests that a common variant in *CCDC93*, p.Pro228Leu (rs17512204), is associated with increased functioning of the CCC complex, an endosomal sorting protein complex that orchestrates LDLR recycling. In agreement with these findings, *CCDC93* p.Pro228Leu is associated with per allele lower levels of plasma LDL-c and a corresponding lower risk of MI and cardiovascular mortality in over 107 000 individuals from the general population (*Take home figure*). These results were validated for LDL-c, risk of both CAD and MI in meta-analyses including from 194 000 to >700 000 participants.

The LDLR has, since its discovery by Goldstein and Brown in the '70s,[21] been recognized as the principal regulator of LDL clearance from the circulation. Its central role in this process is illustrated by the notion that changes in its transcription,[22] internalization,[23,24] stability,[25] subcellular localization, and degradation[26,27] all affect the concentration of LDL-c in blood.

Central to the current study is that Goldstein and Brown also reported that the LDLR can be re-used up to 100 times after its internalization into early endosomes.[28] On the one hand, the re-use of LDLR can be prevented by the binding of pro-protein convertase subtilisin-kexin type 9 (PCSK9) to LDLR. This protein directs the LDLR to the lysosome compartment for degradation,[29] and it has been established that genetic variants in *PCSK9* strongly affect the capacity of the liver to clear LDL from the circulation. On the other

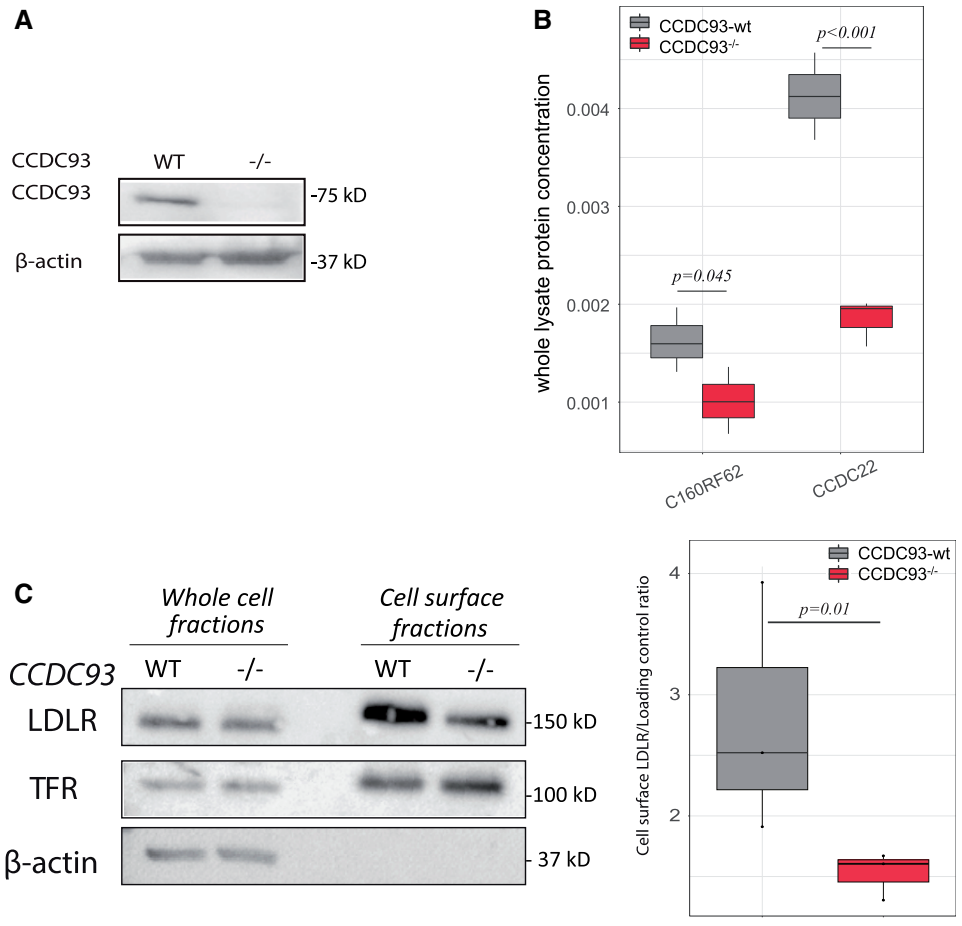


Figure 6 Depletion of CCDC93 impaired low-density lipoprotein-receptor recycling and low-density lipoprotein uptake in liver cells. Huh7 *CCDC93* knockout cells (CCDC93$^{-/-}$) were generated using CRISPR/Cas9. The inactivation of the gene was validated by western blot (*A*). Ablation of CCDC93 (*A* and *B*) resulted in a decreased protein levels of the core components of the CCC (COMMD-CCDC22-CCDC93) complex. (*B*) CCDC22 and C16orf62 were markedly reduced as well as other satellite components of the complex (COMMD5,7,9,10), whereas total low-density lipoprotein receptor levels were not affected. Protein measurements were normalized with housekeeping proteins (beta actin and tubulin). Knockout values were calculated as a percentage of the concentration of proteins in Wt cells. Only statistically significant decreased components of the CCC complex are presented (*B*). Plasma membrane and whole cell fractions were isolated using a biotinylation assay and analysed by western blotting (*C*). Low-density lipoprotein receptor was found to be strongly reduced in cell surface proteome of CCDC93 knockout cells when compare with Wt cells (>50% decrease, $P < 0.01$). Low-density lipoprotein receptor protein quantification was normalized against transferrin receptor as cell surface loading control. Cells were cultured without serum for 16 h and subsequently incubated with DiI-LDL for 2.5 h and imaged by fluorescence microscopy. DiI-LDL uptake was measured and normalized per cell (counted with nuclei DAPI staining). More than 700 cells per condition were analysed and compared. *CCDC93*$^{-/-}$ cells present impaired uptake of fluorescently labelled low-density lipoprotein (DiI-LDL) compare to controls of ~40% ($P < 0.001$) (*D*). Low-density lipoprotein-receptor is the central regulator of plasma levels of low-density lipoprotein cholesterol and can be re-used 100 times after its endocytosis into endosomal network.[28] At the endosomes, low-density lipoprotein receptor is sorted for lysosomal degradation, which is promoted through the binding of proprotein convertase subtilisin-kexin type 9 to low-density lipoprotein receptor,[29] or low-density lipoprotein receptor is recycled back to the plasma membrane for re-use, which is orchestrated by the CCC (COMMD/CCDC22/CCDC93 + C16orf62) complex[30] (*E*). CCDC93, coiled-coil domain-containing protein 93; CCDC22, coiled-coil domain-containing protein 22; C16orf62, chromosome 16 open reading frame 62; COMMD, copper metabolism domain containing; LDLR, low-density lipoprotein receptor; b bellow the detection rate, DiI-LDL 3,3'-dioctadecylindocarbocyanine labelled LDL, DAPI 4',6-diamidino-2-phénylindole. *$P < 0.05$; **$P < 0.01$; NS, not significant.

hand, little is known about the mechanisms that transport the LDLR back to the cell membrane for re-use, and how this may affect plasma LDL-c levels.[30] *Figure 6E* illustrates our current working model.

Endosomal trafficking of different proteins (cargo's) is a long-standing topic in cell biology.[31,32] We have recently taken a next step to study this process *in vivo*.[12,13,30,33] These experimental studies in mice have shown that a loss of proteins of the endosomal sorting machinery causes hypercholesterolaemia and increased atherosclerosis.[12,13] In the current study, we provide evidence that it is also possible to decrease plasma LDL-c in mice through overexpressing

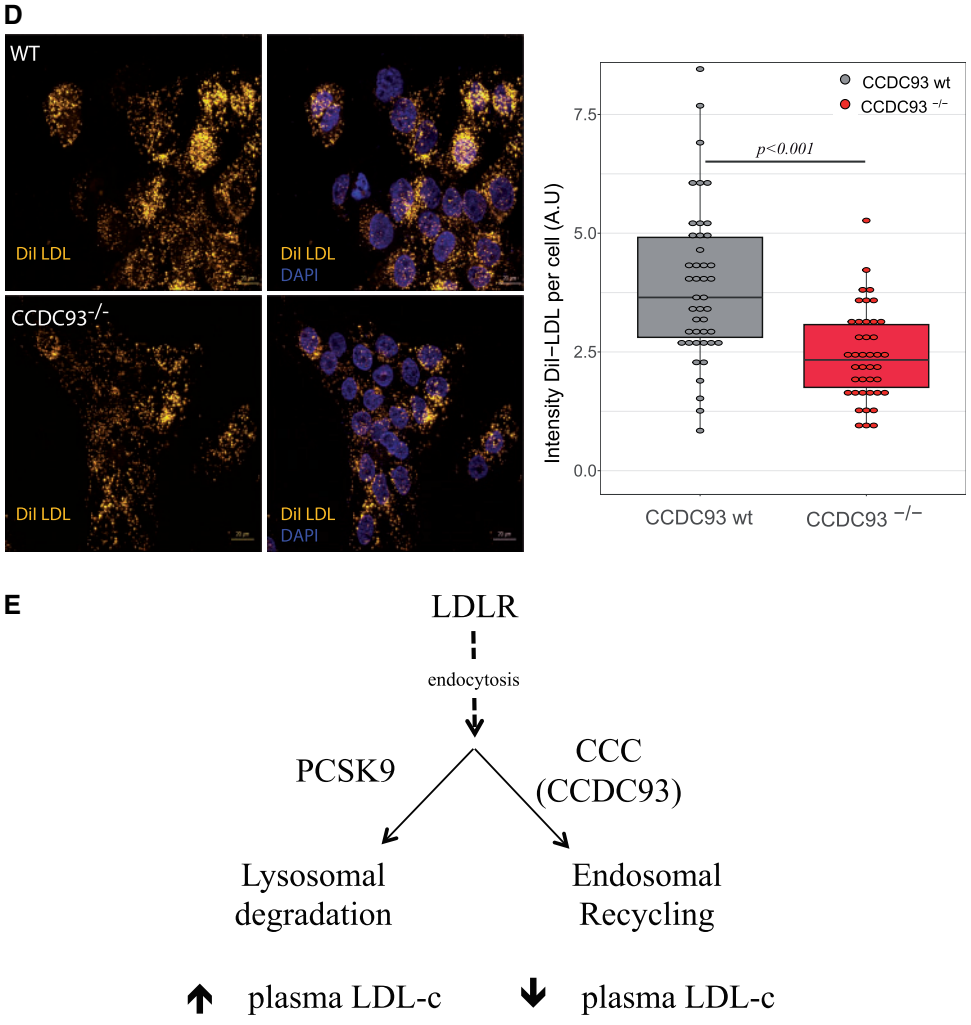


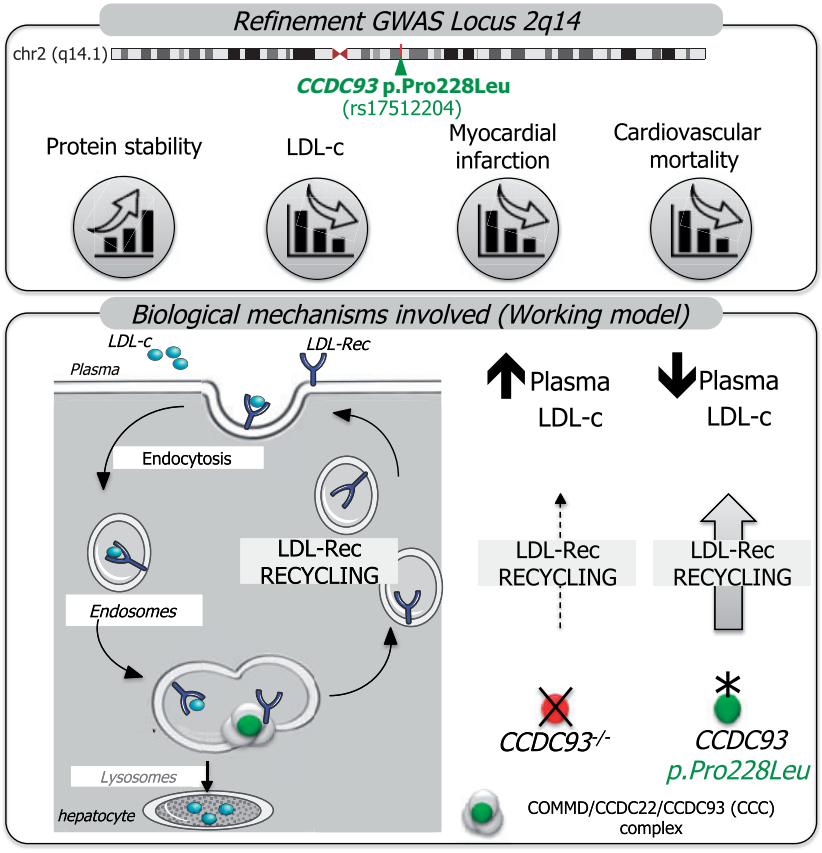
Figure 6 Continued.

wild-type CCDC93 albeit it that the effect is small. Others have shown that over-expression of *SNX17*, another protein involved in the endosomal sorting machinery, also enhances LDL uptake via its positive role on LDLR sorting in cultured cells.[34]

Evidence that the endosomal LDLR recycling may also be relevant in humans was so far restricted to rare loss-of-function mutations in *CCDC22* and *WASHC5*, which cause hypercholesterolaemia but also developmental defects and intellectual disability.[13,35,36] This combined with the notion that a complete loss of components of the endosomal recycling machinery is lethal in mice[12,13,37] indicated that impairment of this pathway is very harmful. In contrast, the current study shows that a common genetic variant, presumably associated with a 'gain-of-function', is associated with cardiovascular risk reduction including a reduction in cardiovascular mortality in the general population.

In meta-analyses, we found similar *CCDC93* p.Pro228Leu per T-allele reductions in LDL-c and risk of CAD in all studies considered, comparable to data reported in a recent consensus statement from the European Atherosclerosis Society Consensus Panel.[1] Taken together, the data suggest that the per T-allele reduction in risk of CAD and MI can be explained by the corresponding observed reduction in LDL-c.

The per T-allele reduction in risk of MI which is a harder endpoint than CHD/CAD was 5% overall, similar between studies (overlapping confidence intervals for all studies), and significant in both CARDIoGRAMplusC4D and in our own study, but not different from unity in the UK Biobank. The most likely explanation for the latter finding is that the participation rate in that study was 5.5% (9 million invited, ~500 000 participants), limiting the representativeness of the study for the UK general population, and possibly leading to a healthy participant bias (the participation rates were 61% and 45%, respectively, in the CCHS and CGPS). The latter is supported by the observation that the proportion of smokers and the proportion with no qualifications—reflecting social position—were considerably lower than in the background population, and that the 5-year mortality rate was several-fold lower than in the UK population as a whole.[38,39] Furthermore, the frequency of MI in the UK Biobank was <2% compared to >5% in the Danish general population, suggesting



Take home figure The refinement of the GWAS locus 2q14 identified *CCDC93* as a novel gene which affects LDL-c plasma levels in man. We show here that the variant rs17512204 (*CCDC93*-p.Pro228Leu), increases the stability of the encoded protein and is dose-dependently associated with lower LDL-c, lower risk of myocardial infarction and lower cardiovascular mortality. The data suggest that the beneficial effects observed are associated with improved functioning of the CCC complex (COMMDs/CCDC22/CCDC93), an endosomal sorting protein complex that orchestrates LDL-Receptor recycling. While CCDC93 ablation reduces LDL uptake as a result of reduced LDLR levels at the cell membrane, overexpression of human CCDC93 decreases plasma LDL-c in mice.

that the UK Biobank represents a low-risk population. Therefore, a common genetic variant, as studied here, which typically associates with a modest change in risk, is at this point in time unlikely to be associated with a measurable risk reduction in the UK Biobank with the current limited follow-up and the low risk at the start of the study.

It is noteworthy that genome-wide association studies of human plasma lipid traits have thus far not pointed at a role for the genes encoding proteins involved in the endosomal recycling process, with the only exception being for *CCDC93* as unveiled in this study. This can be explained by the fact that this process is primarily regulated at the post-translational (protein) level,[12,13,40] which, in turn, supports our hypothesis that the *CCDC93* variant (rs17512204) encodes a protein with improved function.

The current study provides evidence for a role of CCDC93 in human LDL metabolism, cardiovascular disease, and cardiovascular mortality with molecular clues from *in vitro* and *in vivo* experiments that is related to LDLR-mediated LDL-c clearance (*Take home figure*). Proof that loss of function mutations in *CCDC93* may cause familial hypercholesterolaemia in man would strengthen our findings, but mutations in *CCDC22*, another gene encoding for a protein of the CCC complex, have been shown to cause hypercholesterolaemia.[13]

In conclusion, this study shows that large-scale genetic studies can help us find other angles to study LDL metabolism. It has implicated yet another pathway that was already known to control the activity and/or functionality of LDLR, i.e. it is endosomal sorting and recycling in humans. It remains to be seen whether this insight may lead to new tools for pharmaceutical intervention, but it has been shown that it is possible to pharmaceutically target the endosomal-sorting pathway with small molecules.[41]

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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